Inhibition of serotonin-induced mitogenesis, migration, and ERK MAPK nuclear translocation in vascular smooth muscle cells by atorvastatin

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Li M, Liu Y, Dutt P, Fanburg BL, Toksoz D. Inhibition of serotonin-induced mitogenesis, migration, and ERK MAPK nuclear translocation in vascular smooth muscle cells by atorvastatin. Am J Physiol Lung Cell Mol Physiol 293: L463–L471, 2007. First published June 1, 2007; doi:10.1152/ajplung.00133.2007.—The HMG-CoA reductase inhibitors, statins, have pleiotropic effects which may include interference with the isoprenylation of Ras and Rho small GTPases. Statins have beneficial effects in animal models of pulmonary hypertension, although their mechanisms of action remain to be determined. Serotonin [5-hydroxytryptamine (5-HT)] is implicated in the process of pulmonary artery smooth muscle (PASM) remodeling as part of the pathophysiology of pulmonary hypertension. We examined the effect of atorvastatin on 5-HT-induced PASM cell responses. Atorvastatin dose dependently inhibits 5-HT-induced mitogenesis and migration of cultured bovine PASM cells. Inhibition by atorvastatin was reversed by mevalonate and geranylgeranylpyrophosphate (GGPP) supplement, suggesting that the statin targets a geranylgeranylated protein such as Rho. Concordantly, atorvastatin inhibits 5-HT-induced cellular RhoA activation, membrane localization, and Rho kinase-mediated phosphorylation of myosin phosphatase-1 subunit. Atorvastatin reduced activated RhoA-induced serum response factor-mediated reporter activity in HEK293 cells, indicating that atorvastatin inhibits Rho signaling, and this was reversed by GGPP. While 5-HT-induced ERK MAP and Akt kinase activation were unaffected by atorvastatin, 5-HT-induced ERK nuclear translocation was attenuated in a GGPP-dependent fashion. These studies suggest that atorvastatin inhibits 5-HT-induced PASM cell mitogenesis and migration through targeting isoprenylation which may, in part, attenuate the Rho pathway, a mechanism that may apply to statin effects on in vivo models of pulmonary hypertension.

statin; Rho; proliferation

ALTHOUGH LONG RECOGNIZED to participate in physiological responses such as neurotransmission, intestinal motility, and vasoactivity, it is only in recent years that serotonin [5-hydroxytryptamine (5-HT)] has been found to act as a mitogen for a variety of cell types including pulmonary artery smooth muscle cells (PASMCs) (14, 38, 39). The mitogenic effect of 5-HT is initiated either by binding to one or more of the 5-HT receptors (5-HTRs) or through active transport of 5-HT into the cell via the 5-HT transporter (5-HTT) (14). The signaling events triggered by 5-HT leading to mitogenesis are likely to be complex, involving the stimulation of distinct signaling pathways and ensuing pathway cross-talk which are only partially understood.

Several studies indicate a strong association, between 5-HT and certain forms of pulmonary arterial hypertension (PAH), a condition for which there is currently no effective treatment (47). Herve et al. (20) first reported elevation of plasma 5-HT and reduced platelet 5-HT in association with PAH in a patient with familial platelet storage disease, and this was followed by a report of a series of similar patients (21). Subsequently, there was a widely recognized association between appetite suppressants (fen-phen) that share a cellular ligand with 5-HT, and PAH (4), thus further linking 5-HT with pulmonary hypertension. In addition, animal studies identified the Fawn-hooded rat with a spontaneous defect in platelet storage of 5-HT and elevated serum levels of 5-HT to have pulmonary hypertension (53). Moreover, plasma levels of 5-HT are elevated in mice exposed to hypoxia leading to the development of pulmonary hypertension (5), and plasma levels of 5-HT are elevated in humans with idiopathic PAH (33). Part of the cellular pathogenesis of PAH involves arterial smooth muscle remodeling in the form of intimal thickening and muscularization of pulmonary artery and vessels leading to arterial obstruction, and 5-HT is thought to contribute to the pathogenesis of PAH by stimulating the remodeling process (47).

Because of ease of culture and rapid growth, our laboratory has routinely used primary cultured bovine PASMCs to study the action and mechanisms of 5-HT-induced responses on PASMCs. These cells readily undergo DNA synthesis and proliferation in response to low levels of 5-HT (comparable to serum levels) (38, 39). Our previous studies indicated that the ERK MAP kinase pathway (36, 37) and the PI-3 kinase/Akt pathway (42) are required for 5-HT-induced mitogenesis of cultured PASMC. Recent studies also showed that the Rho small GTPase and Rho kinase (ROCK) function are required for 5-HT-induced mitogenesis and that this appears to be mediated via a 5-HTR (43).

Rho is a member of the Ras superfamily of small GTPases and is related to Rac and Cdc42 (26, 30). The most well-characterized Rho proteins are RhoA, B, and C (26). Like other G proteins, Rho cycles between active GTP- and inactive GDP-bound forms, and upstream signals trigger Rho activation and propagation of the signal (26). Rho, and its primary effector ROCK (3), contributes to many cellular functions such as adhesion, contraction, growth, motility, and cytokinesis via their effects on actomyosin filament assembly (3, 26, 30). The role of RhoA in controlling agonist-stimulated smooth muscle cell contractility and nonmuscle cell cytoskeletal organization through its phosphorylation and inhibition of myosin phosphatase with subsequent enhancement of myosin light chain phosphorylation has been extensively characterized (55). Rho/
ROCK is additionally required for cell cycle progression and participates in cell growth (7, 50). During their synthesis, Ras and Rho GTPases undergo posttranslational modifications resulting in the attachment of isoprenoid lipid moieties (1, 6, 62). This isoprenylation step is necessary for the biological activity of these GTPases and enables them to associate with relevant membrane domains (2). RhoA and C are typically geranylgeranylated while Ras proteins are farnesylated; RhoB undergoes both geranylgeranylation and farnesylation (1, 62).

The 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) inhibitor statins (18, 19) have beneficial pleiotropic cardiovascular effects which are thought to extend beyond inhibition of serum cholesterol levels (40, 41, 45). The cholesterol-independent effects of statins are postulated to involve interference with the normal synthesis of isoprenoids geranylgeranylpyrophosphate (GGPP) and farnesylpyrophosphate (FPP) which lie downstream of mevalonate, all of which are metabolic products of the HMG-CoA pathway (41, 45). Early studies showed that statins can inhibit cell proliferation (27), an effect which appears to be isoprenoid dependent. For example, lovastatin dose dependently induces G1 cell cycle arrest in fibroblasts, an effect that is reversed by supplement with mevalonate or GGPP (60), implicating a geranylgeranylated protein such as Rho. Indeed, the suggestion of Rho as a target for the cholesterol-independent effects of statins on responses such as cell transcription, cytoskeletal changes, contractility, and growth is increasingly reported (41, 45).

The use of statins in pulmonary hypertension has begun to be investigated (17, 48, 49, 58). Simvastatin not only prevents monocrotaline-induced pulmonary hypertension of pneumonectomized rats (48), but it also rescues rats from fatal pulmonary hypertension (49), and the reversal correlates with attenuation of pulmonary smooth muscle neointimal proliferation and increased apoptosis (41). Hypoxia-induced pulmonary hypertension in rats is also reduced by simvastatin and the effect does not appear to involve enhancement of endothelial nitric oxide synthase expression (17). Moreover, in a rat model of severe pulmonary hypertension which recapitulates major components of the human disease (induced by VEGF receptor blockade in combination with chronic hypoxia), out of 10 agents tested only simvastatin significantly improved outcome (58). Thus, overall, statins have beneficial effects on pulmonary hypertension in animal models, and an observational case study in patients with severe PAH suggests improvement in certain clinical parameters (31). However, the mechanisms of action of statins leading to beneficial effects in pulmonary hypertension are largely unknown.

Compared with the extensive literature on statin effects on the systemic vasculature, studies of statin effects on the pulmonary vasculature are limited, and only some of these focus on pulmonary artery smooth muscle. Because of the association of 5-HT and SMC remodelling in pulmonary hypertension, we have undertaken this study to evaluate the influence of statins on 5-HT-induced PASMC mitogenesis and migration in cultured cells and to examine the mechanism of action. Atorvastatin (aka Lipitor), a third generation synthetic statin which is widely prescribed, is a member of the lipophilic category of statins (which includes lovastatin, simvastatin) and was chosen for these studies.

MATERIALS AND METHODS

Reagents and antibodies. Atorvastatin calcium in powder form obtained from Pfizer was dissolved in methanol as recommended. All other reagents were from Sigma unless otherwise specified. Mevalonate was dissolved in ethanol; GGPP and FPP were purchased in soluble form. Anti-RhoA monoclonal, anti-MYPT1, and anti-ERK rabbit antibody were purchased from Santa Cruz Biotechnology. Anti-phospho-MYPT1 (Thr696) polyclonal antibody was from Upstate. Anti-cyclin D1, anti-phospho-ERK, anti-Akt, anti-phospho-Akt, and horseradish peroxidase-conjugated polyclonal rabbit antibody were from Cell Signaling Technology.

Cell culture. SMCs from bovine pulmonary artery were isolated as previously described (39) and maintained in RPMI 1640 medium containing 10% FBS, 100 U/ml penicillin/streptomycin. SMCs from passage 3 to 15 were used in studies. Human embryonic kidney (HEK)293T cells from ATCC were maintained in DMEM (Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum and 100 U/ml penicillin/streptomycin. Cells were maintained in a humidified, 5% CO2 incubator at 37°C.

Plasmids. Plasmids encoding activated mutant pSrcx RhoA, RhoB, Rhoc (1), SRE.L luciferase transcriptional reporter (6), and pGEX2T-Rhotekin RBD (51) plasmids were gifts.

Incorporation of [3H]thymidine. SMCs were grown to 80% confluence in 96-well plates and growth-arrested by incubation in RPMI containing 0.1% FBS for 48 h. Cells were then incubated with 1 µM 5-HT or vehicle in the same medium for 20 h, followed by labeling with [methyl-3H]thymidine (20 µCi/ml) for 4 h. For experiments using atorvastatin, GGPP, FPP, and mevalonate, reagents were added 30 min before stimulation with 5-HT. After being labeled, experiments were terminated by aspiration of medium and cells were harvested onto Unifilter 96-well microplates with a Parkard harvester. Incorporation of [3H]thymidine was measured by scintillation counting.

Western blotting. SMCs were grown to confluence in 35- or 60-mm dishes, serum-deprived, and stimulated with vehicle or 5-HT as indicated. Cells were treated with atorvastatin for 24 h before 5-HT stimulation. Whole lysates were collected and resolved by SDS-PAGE as previously described (43). Primary antibodies were used at: anti-RhoA 1:500; anti-phospho-MYPT1, anti-MYPT1, anti-phospho-ERK, anti-ERK, anti-phospho-Akt, anti-Akt, anti-nucleolin, and anti-cyclin D1, anti-phospho-Akt, anti-phospho-MYPT1 (Thr696) polyclonal antibody was from Upstate. Anti-phospho-MYPT1 (Thr696) polyclonal antibody was from Upstate. Anti-phospho-MYPT1 (Thr696) polyclonal antibody was from Upstate. Anti-phospho-MYPT1 (Thr696) polyclonal antibody was from Upstate. Anti-phospho-MYPT1 (Thr696) polyclonal antibody was from Upstate. Anti-phospho-MYPT1 (Thr696) polyclonal antibody was from Upstate. Anti-phospho-MYPT1 (Thr696) polyclonal antibody was from Upstate. Anti-phospho-MYPT1 (Thr696) polyclonal antibody was from Upstate. Anti-phospho-MYPT1 (Thr696) polyclonal antibody was from Upstate.

Rho-GTP pull-down assay. Pull-down assays for RhoA were performed as described (51). Briefly, cells were washed with ice-cold PBS, scraped off the plates in lysis buffer (50 mM Tris, pH 7.5, 1% Triton, 150 mM NaCl, 5 mM MgCl2, 10% glycerol, 2 mMol/l PMSF, 10 µg/ml leupeptin, 20 µg/ml aprotinin) on ice for 20 min. Lysates were centrifuged for 10 min and the supernatant was ultracentrifuged at 100,000 g for 10 min at 4°C. Supernatants were incubated for 45 min at 4°C with GST-RBD beads. Bead pellets were washed four times with ice-cold cell lysis buffer, resuspended in sample buffer, and subjected to SDS-PAGE as described above.

Subcellular fractionation. High-speed fractionation into cytosolic and membrane-rich fractions was performed as described previously (57). Briefly, SMCs (four 100-mm-diameter dishes) were allowed to swell in hypotonic buffer (1 mM Tris, pH 7.5) containing protease inhibitors (1 mM PMSF, 20 µg/ml aprotinin, 10 µg/ml leupeptin) on ice for 15 min. After being scraped, cells were briefly pelleted, resuspended in 300 µl of hypotonic buffer, and homogenized in a Dounce homogenizer with 100 strokes. The homogenate was centrifuged at 10,000 g for 10 min and the supernatant was ultracentrifuged at 100,000 g at 4°C for 1 h. The resultant supernatant was collected as the cytosolic fraction. Pellets were resuspended in 200 µl hypotonic buffer by sonication for 5 x 3 times on ice and saved as membrane...
fraction. Equivalent volume amounts of each fraction were analyzed by Western blotting.

For nuclear fraction isolation, confluent SMCs were growth-arrested in 0.1% FBS RPMI for 24 h. The arrested cells were pretreated with atorvastatin, GGPP, FPP, and mevalonate for 24 h and then treated with 1 μM 5-HT for 5 min. Nuclear extracts were prepared using Nuclear and Cytoplasmic Extraction Kit (NE-PER, Pierce Biotechnology) according to the manufacturer’s instruction.

**Migration assay.** Cell migration was assayed using a modified Boyden chamber system as described in Ref. 10. Growth-arrested cells were trypsinized, suspended with serum-free RPMI, and seeded into the upper compartment of the chamber insert (Costar, Corning, NY). Treatments were added at the same time. After 16-h incubation at 37°C, 5% CO₂, the inserts were removed, fixed with methanol for 15 min, and stained with a crystal violet solution (0.5% crystal violet and 20% methanol) for 1 h. Cells on the upper surface of the filters were removed by gentle wiping. The number of migrating cells was counted at 100 magnification in five fields per well.

**Cell transfection.** Cells at 80–90% confluence were transfected using Lipofectamine 2000 (Invitrogen) as recommended by the manufacturer. Six-hour posttransfection media were replaced with fresh media, and cells were harvested 48 h posttransfection. Backbone vector was added to the transfection mixture to ensure that all groups were transfected with equal total plasmid amounts.

**Transcriptional reporter assay.** Cells were transfected in six-well dishes at 80% confluence with designated plasmids as previously described (13). SRE-luciferase reporter plasmid (6) which encodes a mutant SRE that contains functional SRF binding sites but eliminates the ternary complex factor binding site (22) was used. Each well was also cotransfected with internal control Renilla luciferase expressed from pTK-RL plasmid (Promega) to allow normalization of inducible firefly luciferase values for transfection efficiency, cell viability, and cell number. Forty-eight hours posttransfection, cells were washed with PBS, and Promega reporter lysis buffer was added to each well. Lysates were collected and assayed for luciferase activity by Dual Luciferase Reporter Assay System (Promega) following the manufacturer’s instructions. For each group, the relative luciferase units (RLU) were determined by automatic calculation of the ratio of SRE.L firefly luciferase:Renilla luciferase readings by luminometer. Points were in triplicate.

**Statistical analysis.** Data are expressed as means ± SD. Statistical analysis was performed using the paired Student’s t-test. Differences were considered as significant at $P < 0.05$.

**RESULTS**

**Atorvastatin attenuates 5-HT-induced SMC DNA synthesis.** 5-HT (1 μM) significantly induces [3H]thymidine incorporation in bovine PASMCs. SMCs were treated with increasing concentrations of atorvastatin or vehicle (0.1–30 μM) for 24 h to determine the effect on 5-HT-induced mitogenesis. Atorvastatin, but not vehicle, significantly inhibited 5-HT-induced [3H]thymidine uptake in a dose-dependent manner (Fig. 1A), with 1 μM resulting in significant inhibition, and 10 μM resulting in near background levels. Figure 1B shows that cyclin D1 levels were reduced after 24 h of atorvastatin treatment. Trypan blue exclusion showed no effect of atorvastatin on cell viability in the 0.1- to 10-μM range (not shown), although toxic effects were noted at 30 μM.
Mevalonate and the GGPP isoprenoid reverse atorvastatin-induced mitogenic inhibition. To characterize the statin target, individual components of the HMG-CoA reductase pathway, schematically shown in Fig. 1C, were added together with atorvastatin overnight. The inhibitory effect of 10 μM atorvastatin on DNA synthesis is reversed by mevalonate supplement (100 μM; Fig. 1D). Furthermore, the effect of atorvastatin on thymidine uptake is largely reversed by GGPP (5 and 10 μM). In contrast, FPP (10 μM) had no effect on atorvastatin-induced inhibition, and neither did squalene (not shown).

Atorvastatin inhibits SMC migration induced by 5-HT. We next investigated 5-HT-induced SMC migration using a modified Boyden chamber assay. Figure 2A shows that treatment with 10 μM of the ROCK inhibitor Y27632 inhibited 5-HT-induced SMC migration, indicating a requirement for Rho/ROCK function. Figure 2B shows that atorvastatin at 10 μM effectively attenuated 5-HT-induced migration and that this was reversible by GGPP supplement.

Atorvastatin attenuates 5-HT-induced RhoA/ROCK activation and RhoA membrane localization. The ability of GGPP to reverse the inhibitory effect of atorvastatin suggested modulation of a geranylgeranylated protein such as Rho. On this basis, the status of RhoA was evaluated by measuring the activation state of Rho by the RBD pull-down method (51). 5-HT stimulation led to increased levels of activated GTP-RhoA in PASMCs; moreover, atorvastatin treatment at 1 and 10 μM was found to inhibit 5-HT-stimulated GTP-RhoA formation by ~40 and 70%, respectively (Fig. 3A). As the Rho effector ROCK phosphorylates and thereby inactivates the myosin light chain phosphatase regulator subunit MYPT-1 (32), the effect of atorvastatin on 5-HT-induced MYPT-1 phosphorylation was examined. Treatment with 10 μM atorvastatin significantly inhibited 5-HT-induced phosphorylation of MYPT-1 (Fig. 3B). Activation of RhoA is associated with its translocation from...
the cytosol to the plasma membrane, a process that is thought to be dependent on isoprenylation (1, 2). RhoA subcellular localization was evaluated by high-speed fractionation to isolate membrane-rich and cytosolic fractions. Stimulation of PASMCs with 5-HT (1 μM, 15 min) led to a relative increased level of membrane-associated RhoA and a concomitant decrease in the level of cytosolic RhoA (ratio of membrane-associated vs. cytosolic RhoA induced by 5-HT increased by ~258 ± 98%; Fig. 3C). Twenty-four-hour treatment with atorvastatin dose dependently reversed the 5-HT-induced RhoA membrane association.

**Atorvastatin has no effect on 5-HT-induced activation of ERK and Akt.** Ras/Raf/MEK/ERK signal transduction pathway is recognized to control cellular proliferation in response to extracellular signals (52), and Ras GTPase function requires isoprenoid modification (62). On this basis, we determined the effect of atorvastatin on the phosphorylation status of ERK MAP kinase which participates in 5-HT-induced proliferation of SMCs (36). Phosphorylation of ERK1/2 occurred from 2 to 30 min and peaked at 15 min. Atorvastatin at 1 or 10 μM did not block ERK1/2 phosphorylation (Fig. 4A). To determine whether atorvastatin influences the PI3K/Akt pathway which also participates in 5-HT-induced proliferation of SMCs (42), we examined the effect of atorvastatin on Akt activation. There was no detectable effect of atorvastatin treatment on 5-HT-induced phosphorylation of Akt (Fig. 4B).

**Atorvastatin reduces 5-HT-induced ERK nuclear translocation.** Based on our previous report that Rho/ROCK function is required for 5-HT-induced nuclear translocation of ERK MAP kinase in SMCs (43), we tested the effect of atorvastatin on this process. Overnight, 5 μM atorvastatin-treated bovine PASMCs were briefly stimulated with 5-HT, then lysed and the nuclear fraction was isolated. Figure 4C shows that although atorvastatin does not affect total 5-HT-induced phospho-ERK levels,
it attenuates levels of 5-HT-induced nuclear phospho-ERK. Figure 4D shows that the inhibition of nuclear phospho-ERK levels by atorvastatin is reduced by supplement with mevalonate and GGPP.

**Atorvastatin attenuates activated Rho-induced serum response factor reporter activation.** The effect of atorvastatin on Rho-induced signaling was evaluated using the SRE.L luciferase transcriptional reporter in HEK293 cells. Rho is required for extracellular factor-induced serum response factor (SRF)-mediated transcription which occurs via SRF binding to serum response element (SRE) found in many promoters (22). Figure 5 shows that expression of individual forms of constitutively active RhoA, RhoB, or RhoC stimulates SRE.L reporter induction; and atorvastatin treatment significantly inhibits reporter induction by all three Rho members. Furthermore, in all cases the statin inhibition is reversed by GGPP supplement.

**DISCUSSION**

In this study, we found that atorvastatin dose dependently inhibits serotonin-induced PASMC mitogenesis and migration. In addition, atorvastatin was found to reduce serotonin-induced nuclear translocation of ERK MAP kinase. Many studies address the effects of statins on responses induced by serum rather than by particular stimuli, such as here for 5-HT. While Crespo and Quidgley (9) found that statins inhibit serotonin-induced contraction of coronary arteries, to our knowledge, our study is the first to report effects of a statin on serotonin-induced mitogenesis and migration of any cell type. Martinez-Gonzalez et al. (44) reported that lovastatin inhibits PDGF/EGF/insulin-induced mitogenesis of human PASMCs at 25 μM, while another report (16) found that mevastatin inhibited serum-induced rat PASMC growth at 80 μM. The dose range of atorvastatin at which we observed significant inhibition (1–10 μM) is lower than the effective doses used in these other studies, and even the small inhibitory effect at 0.1 μM atorvastatin (Fig. 1) was significant (*P < 0.018*). The reason for the difference in effective dose between our studies and others may be related, at least in part, to the relatively high activity of atorvastatin (29).

Our finding that 5-HT-induced cyclin D1 levels are reduced by atorvastatin treatment is consistent with reports that statins induce cell cycle arrest (27). While modulation of levels of the cyclin-dependent kinase inhibitor p27kip1 has been associated with regulating vascular SMC growth (15), it is not required for statin-induced PASMC growth inhibition (16), and our studies found no evidence of significant p27kip1 reduction by atorvastatin (not shown). The absence of SMC death/apoptosis in the 0.1- to 10-μM atorvastatin range used in our study is in keeping with other reports that apoptosis is often observed at statin levels above those required for growth inhibition (28), although overall the effects of statins on proliferation and apoptosis appear to be independent of each other (12).

While statins have some HMG-CoA reductase pathway-independent effects, e.g., via directly inhibiting leukocyte function antigen-1 binding (61), our finding that mevalonate supplement reverses the inhibition by atorvastatin indicates that the effect is HMG-CoA reductase pathway dependent. Mevalonate is the precursor of the isoprenoids FPP and GGPP (18) which participate in the isoprenylation of Ras and Rho (1, 62), respectively, and our previous reports show that both Ras and Rho are required for growth of SMCs induced by 5-HT (36, 37, 43). The reversion of atorvastatin-induced inhibition of 5-HT-induced SMC mitogenesis by GGPP presents another example of the pleiotropic effect of statins mediated by isoprenoids. The selective reversion by GGPP, although not by FPP, suggests that the target is primarily a GGPP requiring moiety such as Rho proteins, rather than a farnesylation-dependent protein such as Ras. This is concordant with the report that statin inhibition of PDGF-induced human PASMC growth is selectively reversed by GGPP, although in this report a link with Rho was not made (44). Normally GGPP is synthesized from the combination of FPP and isopentenyl pyrophosphate (IPP); however, as statins block the pathway upstream of all these moieties, under statin action GGPP cannot be synthesized even upon FPP supplement, as IPP is not available (18).

However, reversal of inhibition with GGPP implies that such a mechanism does not play a major role in the effects observed here.

Our finding that 5-HT stimulation leads to increased levels of SMC GTP-RhoA and that atorvastatin inhibits 5-HT-induced GTP-RhoA formation is consistent with our previous study showing that expression of dominant negative RhoA or treatment with the Y27632 ROCK inhibitor abrogates DNA synthesis (43), indicating that the RhoA/ROCK pathway is necessary for 5-HT-induced SMC proliferation. Furthermore, our results are concordant with at least two other reports that statins negatively regulate the GTP loading of RhoA in monocytes (25) and lymphocytes (11). Our finding that atorvastatin also prevents 5-HT-induced activation of SMC ROCK, as measured by phosphorylation of MYPT-1, the ROCK target, is concordant with the decreased GTP-Rho levels upon treatment.

Isoprenylation of small G proteins such as Rho serves to anchor the protein to the membrane necessary for G protein activation and signaling (62). Hence, the reduction in 5-HT-induced GTP-RhoA levels by statin likely reflects the inability of prenyl-deficient Rho to become membrane associated and thus activated in response to 5-HT. In support of this, we

Fig. 5. ATV inhibits Rho-induced transcriptional reporter activation, and GGPP reverses the effect. HEK293 cells were transfected with activated pEEX/V-RhoA, B, or C L63 plasmids, and SRE.L luciferase reporter along with control luciferase reporter, and treated with vehicle or ATV (10 μM) without or with 10 μM GGPP. Cells were lysed the next day and luciferase levels were measured by dual luciferase assay (n = 3). Results shown are representative of 3 separate experiments. *P < 0.05 for vehicle vs. ATV. *P < 0.05 for ATV and GGPP vs. ATV alone.
observed that 5-HT treatment results in the translocation of PASMC RhoA from the cytosol to a membrane-rich fraction and that this translocation was markedly reduced by atorvastatin treatment. Similarly, statin inhibition of RhoA translocation to the plasma membrane has been observed in breast cancer and endothelial cells (12, 34); this may be a consistent effect of statins, which is as important as modulation of Rho activity per se. This is illustrated by the report that while statin treatment of glial cells results in impaired cytoskeletal responses and Rho membrane localization, levels of GTP-Rho are paradoxically increased (8). Translocation of Rho to the membrane is critical for activation of downstream effectors such as ROCK, hence this result is also concordant with the reduced phospho-
MYPT-1 levels in atorvastatin-treated PASMCS.

Our previous studies showed that multiple signaling pathways such as PI3K/Akt, ERK, and RhoA/ROCK pathways are involved in 5-HT-induced proliferation of SMCs (36, 37, 42, 43). In our studies here, atorvastatin treatment did not prevent ERK activation, implying that atorvastatin has no effect on Ras signaling, consistent with the lack of effect of FPP supplement on mitogenesis, and concordant with other studies de-emphasizing Ras as the major target of statin-induced growth inhibition (12). Our result showing that PI3K/Akt pathway is not inhibited by atorvastatin treatment is in keeping with the report that statin had no effect on ERK or Akt activation in saphenous vein smooth muscle (59), although the reported effects of statins on the PI3K/Akt pathway can differ depending in part on the cell type/stimulus/statin dose. Overall, our results for PASMC mitogenesis highlight the selectivity of statin action for a GGPP-dependent target such as Rho.

Although there was no change in 5-HT-induced ERK MAP kinase activation by atorvastatin, we investigated 5-HT-induced ERK MAP kinase nuclear translocation based on our previous findings that Rho/ROCK is required for nuclear translocation of activated ERK. The finding that atorvastatin reduces the levels of nuclear phospho-ERK is a novel finding and suggests a previously unrecognized mechanism by which statins may inhibit cell growth, given the importance of ERK MAPK in the mitogenic response. Further investigation of this response showed that it can be reversed by mevalonate or GGPP, a finding that is concordant with our previous report (14) that Rho/ROCK plays a role in this translocation process.

Pulmonary artery remodeling associated with pulmonary hypertension may involve aberrant smooth muscle migration as well as proliferation (47), and 5-HT stimulates pulmonary (10) and systemic (46) arterial SMC migration. Our finding that 5-HT-induced migration of PASMCS, in part, involves ROCK and is inhibited by atorvastatin is concordant with the concept that migration requires Rho/ROCK-mediated cell tension via stress fiber formation and focal adhesion assembly (24).

The use of the Rho-sensitive SREL transcriptional reporter showing that atorvastatin inhibits over 50% reporter activation even by constitutively activated RhoA, B, or C implies the physiological importance of Rho prenylation for biological function, regardless of the GTPase activation state. The strong inhibition of RhoB-induced signaling is in keeping with other reports showing that statins induce a dramatic accumulation of RhoB which is defectively prenylated (56). Whereas RhoA and RhoC have broadly similar biophysical properties with relatively stable half-lives, RhoB is a more divergent short-lived protein (26), and its rapid turnover may render it more sensitive to statin effects via modulation of isoprenoid synthesis. This result also illustrates the general finding that isoprenylated G proteins do not respond uniformly to mevalonate depletion (23), even when they are closely related as in the case of Rho proteins. A contrasting report found that isoprenylation of RhoB was not necessary for SRE-mediated transcription, although it was necessary for RhoB transforming activity (35). However, this study was not based on statin use, hence the differences are likely due in part to use of different reagents. The biological functions of RhoB are divergent from those of RhoA and C and have been linked with endocytosis and apoptosis control (26). The precise contribution of the different Rho isoforms to PASMC proliferation and migration is not known and warrants further study. Reversal of statin inhibition of Rho signaling by GGPP demonstrates that geranylgeranylation is critical for Rho signaling function.

In many of our experiments, we observed that overnight statin treatment resulted in elevation of total cellular RhoA levels, as is most evident in Fig. 3, A and C. In addition to participating in Rho localization, Rho isoprenylation contributes to the normal regulation of Rho protein levels, and inhibition of isoprenylation leads to decreased degradation resulting in the accumulation of inactive Rho in the cytosol (23, 56). Thus the increased levels of cytosolic SMC Rho observed here are concordant with these reports and likely reflect impaired degradation of the unmodified protein leading to its accumulation.

Overall, our findings that atorvastatin inhibits 5-HT-induced SMC mitogenesis and migration are concordant with the inhibition of neointimal smooth muscle accumulation by simvastatin seen in hypertensive rats (49). The growth inhibition by atorvastatin is isoprenoid dependent, and in particular GGPP rather than FPP. Atorvastatin targets the Rho/ROCK pathway shown here to be activated by 5-HT, consistent with the GGPP dependency of the effect. However, our results do not rule out an effect on another geranylgeranylated protein(s). Additional studies are needed to further characterize mechanisms of statin action on pulmonary cell function and in animal models of pulmonary hypertension.

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